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Experimental Marine Microcosm Test Protocol and Support Document. Measurement of the Ecological Effects, Fate and Transport of Chemicals in a Site-Specific Marine Ecosystem

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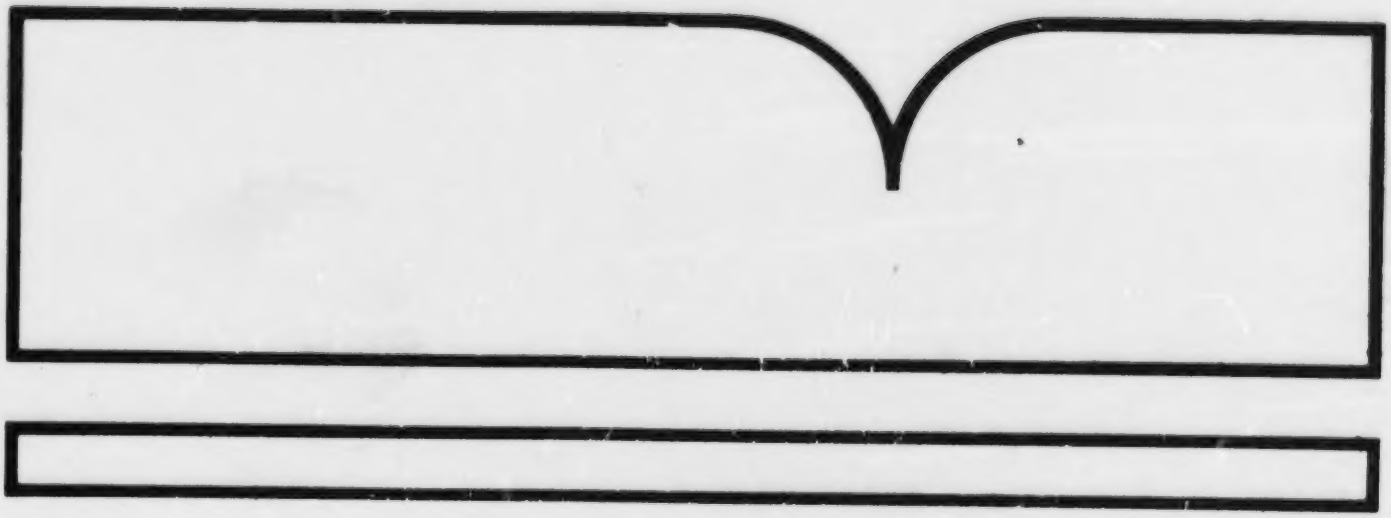

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EXPERIMENTAL MARINE MICROCOSM TEST PROTOCOL AND SUPPORT DOCUMENT
Measurement of the Ecological Effects, Fate and Transport of Chemicals in a Site-Specific Marine Ecosystem

by

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Experimental microcosms designed to simulate major physical, chemical and biological complexities of site specific, coastal marine ecosystems are described.

Procedures are presented which enable the investigator to determine the fate, ecological effects and transport for "new" and "existing" chemicals.

Experimental designs are recommended to ensure valid testing of various dose-response relationships. In addition, certain criteria and conditions are identified as critical to environmental assessment of toxic chemicals.

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TABLE OF CONTENTS

	Page
(A) PREFACE	1
(B) TEST PROTOCOL	4
1. Scope and Purpose	4
2. Definition of Natural System	4
3. Test Substance	6
4. Microcosm Facility	8
5. Test Procedures	13
5.1 Test Purpose	13
5.2 Assumptions	13
5.3 Test of Assumptions	14
5.4 Use of Test Results	14
5.5 Preliminary Testing	15
5.6 Definitive Test	17
5.7 Waste Disposal	26
6. Data Analysis	27
7. Test Protocol Bibliography	33
8. List of Figures	35
(C) SUPPORT DOCUMENT	36
Congressional Mandate	36
Purpose of the Support Document	36
Rational for the Use of Microcosms	36
Rational for Microcosm Design	37
Rational for Microcosm Materials	38
Rational for Test Procedures	38
Test Water Collection and Distribution	38
Compound Addition	39

	Page
Sampling	40
Ecological Effects Chemistry	
Cleaning	42
Data Analysis	43
Restrictions of Test Protocol	43
Support Document Bibliography	45

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PREFACE

(A)

Rational and Constraints of the Marine Microcosm Test Protocol

Commercial chemical companies are required, under the Toxic Substances Control Act, to submit information about new and existing chemical compounds. This information is used by the Environmental Protection Agency to develop an environmental and/or risk assessment for each chemical. In the marine environment, the assessment would include effects of the chemical on the receiving body or ecosystem and the potential for indirect human exposures through the consumption of marine organisms. Such information is usually derived from simple test systems. For example, components of the ecosystem (e.g., single species) or processes (e.g., water solubility, biological breakdown, etc.) are isolated and either exposed to or measured in the presence of the chemical of interest. These data are then used to develop an environmental assessment. However, such assessments have been subject to question on three major grounds. First, the data base is usually confined to the measurement of only a few variables. Second, the validity of these data is tenuous since the physical, chemical and biological complexities of the natural ecosystem are not simulated. Third, the ability to generate a meaningful and accurate prediction of the fate, transport, and ecological effects of the chemical in a natural ecosystem, using these data, is highly questionable.

The Microcosm Test Protocol described herein is designed to overcome many of the concerns associated with environmental assessments based upon existing simple (single species and processes) test systems. Specifically, undisturbed, natural pelagic and benthic communities are

coupled within a single system whose physical (e.g., temperature, water turnover, water turbulence, etc.) and chemical (e.g., salinity, oxygen, sediment pore water, etc.) conditions are equated to that in the natural system being simulated. The ability to simulate a natural ecosystem, demonstrated by comparing the experimental controls to the field, provides us, we believe, with data that are far more applicable to predictive environmental assessments than those derived from more simple tests. Furthermore, since the information generated via microcosm experiments is integrated at the system level of organization, quantitative relationships can be determined for factors such as chemical exposure concentrations and transport rates to measured body burdens, ecological effects and input concentrations/loadings. The acquisition of such a diverse information base in a single test makes this experimental approach to the development of an environmental assessment highly cost effective when compared to the summed capital, operating and professional interpretive costs of all the isolated tests refered to above.

Finally, some words of caution in the application of this test protocol are necessary. The data and ensuing chemical assessment derived from this procedure are constrained in two primary areas. (1) Because of the size of the microcosm tank, macrofauna such as fishes and large crustaceans have been excluded. As a result, some economically important species cannot be tested in these systems. In addition, macrofauna exclusion could possibly affect the responses of some of the measured ecological effects and chemical fate variables. Whether macrofauna significantly influence such variables (phytoplankton, zooplankton, nutrients, chemical distribution, etc.) for different ecological systems, particularly within the experimental time frame of 30 days, remains to be established. (2)

The microcosm test system was developed from a temperate Northeast (U.S.) estuarine ecosystem where intertidal and reef (e.g., oyster, coral, etc.) subsytems are either small in area or missing. The applicability of this protocol to other site-specific marine ecosystems where either shallow conditions and/or major reef subsystems exist has yet to be determined.

TEST PROTOCOL

1. SCOPE AND PURPOSE

1.1 The standards of this subsection are designed to develop site specific data on the chemical fate and ecological effects of "photo-stable" (see Section 5.1.1. for definition) chemical substances and mixtures ("test substances") subject to test regulations under the Toxic Substances Control Act (TSCA). The United States Environmental Protection Agency (EPA) will use these data to assess the hazards a chemical may present to the marine environment and potentially, human health, through uptake and accumulation in human food species.

2. DEFINITION OF A NATURAL SYSTEM

2.1 The natural marine system (designated as "natural system") at a particular geographic location consists of a coupled water column and benthic subsystem. Within both the water column and benthic subsystems, certain physical and chemical conditions, and biological properties exist. These conditions and properties are simulated in laboratory microcosms as described in Section 4.2 and 5.6.2.

2.1.1 The physical and chemical conditions of the natural system are defined in terms of its:

(1) Boundaries - The boundaries of the natural system are delineated by the maximum excursion of water above and below a selected point during one complete tidal cycle.

(2) Light - The light regime of the natural system is

characterized by: the photoperiod, the best estimate of average monthly incident radiation (total incident radiation per day divided by photoperiod), the average seasonal water extinction coefficient, the average daytime vertical light intensity for the entire water column, and the depth at which the average light intensity occurs.

(3) Sea water - That fluid existing within the boundaries of the natural system.

(4) Sea water turbulence - The average water motion realized for the entire water column of the natural system during the period of experimentation.

(5) Sea water turnover rate - The time required for one complete water turnover or exchange within the defined boundaries of the natural system.

(6) Ratio of benthic surface area to sea water volume - The ratio obtained by dividing the calculated benthic surface area of the natural system by the best estimate of water volume of the system.

(7) Sediment - The bottom substrate existing, at the mean water depth, within the boundaries of the natural system.

(8) Sea water flow rate over sediment surfaces - The average tidal velocity over the bottom as measured in the natural system.

2.2 Biological Conditions and Descriptions

2.2.1 The biota of the natural system is characterized by the organisms in the water column and benthic subsystem.

(1) The water column is characterized by the numbers and species

composition of phytoplankton, zooplankton, and transient larval forms found in the sea water at the designated collection site at a mid-tide. The water column, with its assemblages of biota, is obtained from the natural system by the methods described for filling the microcosms (Section 5.6.2(1)).

(2) The benthic subsystem is characterized by the sediment type and the structural composition of the benthic community, i.e., numbers, species composition, and feeding types of organisms collected frome the benthic boxes (Section 4.2.1 (2 & 3) and retained on a 0.5 mm screen.

(3) If the natural system has more than one distinct benthic community, then those having direct routes to human consumption and/or are of significant economic and aesthetic importance should be considered for experimental use. If some benthic communities contain species which are known to be more sensitive to environmental contaminants than others, then these communities should also be considered.

3. TEST SUBSTANCE

3.1 Information Required

3.1.1 A detailed description of the test substances (radiolabled and unlabeled) including its source, lot number, composition and physical chemical properties must be provided. It is recommended that the test substance be radiolabeled to enable the rapid, accurate measurement of the original test material and potential transformation products, both within and outside the microcosm.

Other applicable analytical techniques (Section 5.6.4(8)) should also be utilized.

3.1.2 The degree of purity and the identification and quantification of any impurities must be established.

3.1.3 It is extremely important to have a knowledge of, or a best estimate of the test substance's mode and form of entry into the marine environment. Specifically, does the test substance enter by atmospheric or aqueous sources? Is the test substance in the form of a gas, liquid, or solid? Is the test substance in association with other materials such as a carrier or solvent mixture? As will be discussed in Section 5.5.1, the extent of such information will determine the method by which the test substance is added to the experimental microcosms. In addition, the test substance(s) photo-oxidation potential, solubility in sea water (within the salinity and temperature range of the natural system), solubility in potential carriers (if appropriate as per Section 5.5.1 (b)), vapor pressure, specific gravity (if a liquid), and octanol:water partition coeficient should be quantified.

3.2 Methods must be described for collecting and measuring the test substance and potential transformation products in and/or on the: gas phase, sea water (both particulate, 0.22 microns, and dissolved, 0.22 microns), surface film, sediment, biota (both whole animal and specific tissues if necessary), and microcosm (glass) surfaces.

4. MICROCOSM FACILITY

4.1 The microcosm facility (Figure 1) is designed to simulate relevant physical, chemical, and biological conditions existing in the natural system described in Section 2. It is composed of two basic parts: (1) the support equipment (room, waterbath, light and turbulence fixtures, test compartments, and an air evacuation system) and (2) the experimental microcosm (tanks, paddles, benthic cylinders, pumps and pump air supply).

4.1.1 Support Equipment (See Figure 1)

(1) All testing must be performed in a room that eliminates any light source other than that provided by the microcosm facility.

(2) The microcosm tanks must be held in a waterbath of sufficient dimensions and flow rates as to provide uniform test temperatures within all treatment conditions and maintain those temperatures within 1^{o} C of the natural system.

(3) A canopy above the waterbath should hold sufficient fluorescent lighting to provide an even light distribution at the desired light intensity, as measured by a photometer, over the water surface of water all the experimental microcosms. Light is provided by "Cool White" fluorescent bulbs, and the photoperiod controlled by a 24 hour timer.

(4) Turbulence is provided by a system that consists of an electric motor, chain drive, mounts and drive shafts. This system is mounted on the canopy with the drive shafts extending through, directly above each microcosm tank (Figure 1). The paddles are bolted to the drive shafts. The speed and direction of the

paddles are controllable. The direction is automatically reversed at regular intervals (i.e.,30 seconds) by an electronic controller (Figure 2).

(5) The open area between the canopy and waterbath is enclosed and sealed with plexiglass. The front plexiglass covers are removable to facilitate setting up and filling the microcosm tanks. Plexiglass panels are also mounted transversly within the waterbath/canopy module, extending approximately five cm. below the water surface, to provide relatively airtight compartments. The number of test compartments is dependent upon the number of test treatment conditions. All tanks within a given test compartment must have the same concentration of the test substance. Access to the microcosm tanks during a test is via hinged ports mounted in the front plexiglass cover.

(6) A slightly negative air pressure is maintained within each test compartment. An exhaust fan draws air from the test compartments through a PVC pipe manifold. The exhaust air is passed through activated charcoal filters and vented through a stack outside of the laboratory building (see Figure 1).

4.2 Experimental Microcosms

4.2.1 The experimental microcosms are designed to provide a multi-trophic level experimental chamber that has coupled pelagic (water column) and benthic communities similar to those existing in the natural system.

(1) The microcosm tanks are 140 liter, cylindrical, round bottomed, "hard" glass (semi-solid @ 600°C) containers. The tanks are

held in position by a plywood "collar" mounted in the water bath trough and encircling each tank approximately 15 cm below the rim.

(2) The benthic boxes are thick walled glass cylinders, open at both ends, and are approximately 30 cm long (Figure 3). The inner diameter of the benthic cylinder must be such that the microcosms sediment surface area to water volume ratio equals that of the natural system (Section 2.1(6)).

(3) The benthic cylinders are used by divers to collect undisturbed sediment cores from the prescribed portion of the natural system 2.1.1 (7) and 2.2.1 (3). The depths of the core should be equal to or greater than the depth of biological habitation or activity but not exceed 25 cm. The bottoms of the cylinders (cores) are sealed, prior to installation in the microcosm tanks, by placing them in slightly larger diameter crystallization dishes (Figure 3). The cores, at this point, should be watertight i.e., the water overlying the sediment core will not flow downward between the sediment periphery and the glass cyclinder. If shells and/or other debris, biologically derived tunnels, etc. preclude this condition, then such cores should be rejected. The length of time between collection of the cores and initiation of the test should be as short as possible. If there is an interim period that should exceed one to two hours, they can be held in the laboratory, submerged in flowing sea water.

(4) Water flow over the sediment core in the microcosm is provided by an all glass, air displacement pump (Figure 3). The dimensions of the pump should be sufficient to provide a flow comparable to the average bottom water flow rate of the natural system. The pump

is positioned in the tank adjacent to the benthic core (Figures 1 & 3). The benthic core is mounted in the microcosm tank so that the cylinder overflow port is approximately five cm. above the tank water level. The cylinder and pump is supported by a glass rod platform that attaches to the rim of the tank. Water from the microcosm tank enters the pump through a small (2 cm) hole in the bottom. When air pressure is applied to the pump a thin circular glass coverslip inside the pump is forced downward and seals the bottom hole. This forces water in the pump through a connecting glass tube and into the benthic cylinder. The water entering the benthic cylinder is directed into several horizontal streams by a glass diffuser in order to minimize sediment disturbance and resuspension. The air continues to force water out of the pump, and concurrently a controller (Figure 4), until a float switch in the controller reaches a lower level. This stops the air flow, allowing water to re-enter the pump from the microcosm tank. When the float switch reaches an upper limit, the air resumes flowing and the cycle is repeated. Water overflows from the benthic cylinder back into the microcosm tank through a small hole located approximately five cm. above the sediment level. The rate of water flow should approximate the mean tidal velocity expressed at the sediment surface of the natural system (Section 2.1.1(8)).

(5) The light intensity over the sediment surface should approximate that incident in the bottom meter of the natural system. This is accomplished by placing a light filter or cover over the top and sides of the benthic cylinder (Figure 3). See Section 5.6.2(6)

for establishment of water column light intensity.

(6) Turbulence is generated by glass paddles that are connected to the drive shafts described in Section 4.1(4) and Figures (1&2). The paddle consists of a vertical shaft with two rows of smaller diameter glass shafts fused to it radiating horizontally outward (Figure 2). The length and number of arms are dependent on the microcosm tank, benthic cylinder and pump configurations, and the level of turbulence desired. The turbulence level may also be controlled by regulating the speed of rotation. The motor controller (Figure 2) automatically reverses the direction of rotation every 30 seconds to prevent the formation of a vortex. The speed of paddle rotation is adjusted so that the dissolution rate (described as weight loss/time) of pure gypsum ($CaSO_4$) blocks (1.5cm x 2.5 cm x 1.0 cm), distributed throughout a microcosm tank is statistically equivalent to the dissolution rates of gypsum blocks measured in the natural system during the season of the experiment. Dissolution rates should be measured and water turbulence adjusted prior to each experiment.

(7) There are two pieces of test equipment that primarily serve an analytical function. These are:

(1) The glass rods. Three millimeter diameter glass rods are cut into five centimeter lengths and bent into a U-shaped configuration. These rods, suspended from the tank rim below the water surface, are periodically removed, placed in scintillation vials, and analyzed to provide an estimate of the amount of test substance adsorbed onto the glass surfaces of the microcosm.

(2) The surface film protector. If the test substance forms a

surface film that could contaminate water or glass rod samples as they are removed from the microcosm, then a small 'surface film protector' must be installed in order to allow collection of non-contaminated sub-surface samples. The protector consists of a "small" (<1% of microcosm water surface) glass cylinder (4.5 cm diam. x 6 cm long) that is suspended from the side of the tank and extends several centimeters above and below the surface of the water (Figure 5). Any surface film within the cylinder can be temporarily removed by wicking with an absorbent/adsorbent material, e.g., cellulose acetate filter pad. Glass rod and water samples can then be taken through the cylinder with minimal surface film contamination.

(8) All test equipment and sampling devices that come in contact with the sea water and test solutions must be pre-treated by washing with detergent, rinsing with deionized water, and heated in a muffle furnace to 450°C for four hours prior to use.

5. TEST PROCEDURES

5.1 Test Purpose

5.1.1 The purpose of the experimental ecosystem (microcosm) test is to determine the chemical fates and ecological effects of various "photostable" chemicals within a site specific marine or estuarine ecosystem. "Photostable" chemicals are defined as those compounds which exhibit minimal photodegradation under natural conditions within a 30 day period.

5.2 Assumptions

5.2.1 The extrapolation of perturbation or test substance results

derived from the experimental microcosms to the natural system is based on the correspondence of unperturbed biological events expressed in the experimental control tanks and in the natural system at the time of the experiment. For those biological events that are statistically equivalent ($\alpha = 0.05$), it is assumed that the perturbation behavior observed in the experimental tanks would also be realized in the natural system, if similarly perturbed.

5.2.2 If Section 5.5.1 (b) applies, then it is assumed that the arbitrarily chosen mode and form of entry of the test substance into the microcosms will be sufficiently equal to the actual entry into the natural system.

5.3 Test of Assumption 5.2.1

5.3.1 Chemical fate and ecological effects of the test substance can be ascertained in the experimental microcosms and related to the expected perturbation behavior in a natural system provided the levels of all relevant independent variables, either simulated and/or realized in the microcosms, are equal to those in the natural system at the time of the test.

5.4 Use of Test Results

5.4.1 The data generated from these microcosms will be used to better understand the potential fate, transport and ecological effects of the test substance in marine ecosystems. The assessment should discuss the potential ecological effects and possibility of indirect human exposures associated with the mode and form of release of the test substance in a specific marine ecosystem.

5.5 Preliminary Testing

5.5.1 Input of test substance: mode and form of entry.

(a) If the mode (i.e., atmospheric and/or aqueous) and form (i.e., gas, liquid or solid) of entry of the test substance into the marine environment is known as with existing chemicals or predictable as with "new" chemicals under TSCA, then similar mechanisms of entry will be simulated in the experimental microcosms. If the only mode of entry is the atmosphere, then this protocol is not applicable at this time. However, if the mode of entry is partially or completely aqueous, then the test substance in its realistic form will be added to achieve nominal water concentrations as per Section 5.5.2, or (b) if the mode and form of entry of the test substance into the marine environment is unknown, then the mode of entry into the microcosms will be aqueous.

Under item (b) above, the form of entry will depend upon the solubility of the test substance in sea water. If the test substance is insoluble in sea water, then "appropriate" carriers will have to be used. An appropriate carrier would completely dissolve the test substance and result in a uniform water column distribution of the test substance at the start of the experiment (see definitive test, Section 5.6). An "inappropriate" carrier would cause the test substance to be distributed within one compartment of the microcosm in a short period of time. Using DEHP for example, were pentane the carrier, then the majority of the test substance, at the start of the experiment, would be distributed at the surface of the water column. With methylene

chloride, the test substance would immediately be found on the bottom of the microcosm tank and subsequently on the sediment surface (see Section 5.6.2 (9)). Acetone is an example of an appropriate carrier and is recommended where applicable. Homogeneous water column concentrations of the test substance are to be achieved as per Section 5.5.2 at the start of the experiment.

5.5.2 Input of Test Substance: Desired exposure concentrations. Nominal exposure concentrations of the test substance within the microcosms should be based upon field exposures and should vary by at least two orders of magnitude.

For "new" chemicals under TSCA, exposure concentrations will have to be predicted based upon either (a) mathematical models, (b) analogue chemicals or (c) a combination of (a) & (b). If such information are unavailable or of questionable validity concentrations of 1, 10 & 100 ug/l will be used (for rational see Pg. 42, Restrictions of Test Protocol, Paragraph 2).

For "existing" chemicals under TSCA, exposure concentrations in the microcosms should be based upon field monitoring data. If field data are unavailable, the determination of exposure concentrations will be identical to the procedure outlined above for "new" chemicals

5.5.3 Sampling and measurement

Preliminary small scale tests should be performed to determine measurement techniques and the most efficient sampling program. These tests should develop information regarding methods for capturing and measuring the test substance and potential

transformation products in the water, gas, sediment, tissues, and on glass surfaces.

5.5.4 Light Intensity

Preliminary tests should be performed to establish the proper light intensity over the microcosms. These tests should be done with all the microcosm equipment and facilities (i.e., waterbath, tank, paddle, benthic box and pump) in place. The tests should be performed at several light levels, based upon conditions listed in Section 2.1.1 (2), with photoperiod equal to ambient conditions, for at least 14 days. Those light levels producing phytoplankton abundances that are statistically equivelent to the natural system should be used in the definitive test.

5.6 Definitive Test

5.6.1 The definitive test will consist of dosing the microcosms with the test substance in the mode, form and concentrations established in Sections 5.5.1 & 5.5.2. Each concentration should have at least five replicates.

(1) There should be at least five replicate control microcosms. If a carrier is used (Section 5.5.1 (b)), then two microcosms with just carrier added (carrier controls) and three as sea water (carrier-free) controls will be used.

(2) The definitive test will consist of:

(a) Addition of the test substance to the water column of the experimental microcosms at the start of the test.

(b) Performance of the test for 30 days, during which the continued addition of the test substance to the microcosms is

consistent with a known/ expected/projected release pattern, or is performed immediately following a water exchange, in sufficient volume to bring only the replacement water up to the initial concentration. It is recommended that this test be conducted during the temperature extremes of an annual cycle.

(3) During the 30 day exposure period, one tank from each treatment condition, except controls, must be sacrificed (at days 10 and 20) and subjected to comprehensive sampling (Section 5.6.6). Following the exposure period (day 30), all remaining microcosms should be similarly sacrificed and sampled.

5.6.2 The microcosm test system is placed in operation as follows:

(1) The test water is collected from the natural system at mid-tide by hand bucketing or non-destructive pumping, (diaphragm pump) and transported to the test facility in glass tanks. If the natural system is vertically stratified in terms of temperature and/or salinity, the initial filling water should be a mixture (average) of water collected from various depths. Subsequent collections for water exchange (Section 5.6.2(10)) and biological sampling (Section 5.6.5) are performed in the same manner.

(2) During the filling process, the water is distributed equally among the microcosm tanks until the prescribed volume is reached.

(3) The benthic cylinders and crystallization dishes containing the sediment cores (Section 4.2.1(2&3)) are placed in the tanks (Section 4.2.1(4)) and any disturbed sediment allowed to settle (approximately 30 minutes).

(4) The benthic pumps are placed adjacent and connected to the benthic cores (Figure 2). Air lines (Figure 1) supplying low

pressure air are attached to create the desired water flow rates over the sediment surface (Section 4.2.1(4)).

(5) The paddles are installed. The speed of rotation is established (see Section 4.2.1(6)) to generate a turbulence level equivalent to that in the natural system.

(6) Light intensity at the surface of the water in the tanks is controlled by adjusting the shading of fluorescent lights to achieve levels as determined in Section 5.5.4. Photoperiod is set equal to ambient conditions.

(7) Water flow in the water bath is adjusted to provide temperature tracking within $1^{o}C$ of the natural system. Flow rates must also be sufficient to maintain all experimental tanks within $1^{o}C$ of each other.

(8) After the disturbed sediment in the benthic cylinders settles, the low pressure air to the benthic pumps is turned on and water flow to the benthic cylinders begun at an average rate equal to the average tidal flow over the benthic substrate in the natural system. (Sect. 4.2.1(4)).

(9) Any resuspended sediment that settles on the bottom of the microcosm tanks at any time during the experiment should be collected with a tubing pump and replaced into the benthic cylinders.

(10) Water turnover volume and frequency is established to match the exchange rate of the natural system (Section 2.1.1(5)). Water exchange in the microcosm tanks should be scheduled at least three times a week and should coincide with the biological and chemical sample collections. The discreet volume of water

removed during the water exchange equals the calculated amount minus that volume removed during biological and chemical sampling.

(11) If a surface film of the test substance is formed, a portion of that film must be removed during each water exchange to simulate natural transport. The area of surface film to be removed by wicking with filter pads or other suitably absorbent material is defined by:

$$\text{Area } (cm^2) = \frac{\text{water turnover volume/turnover day}}{\text{total microcosm water volume}} \times \text{benthic surface area}$$

5.6.3 Test Substance Addition

5.6.3(1) Carriers

(1a) If a carrier other than sea water is used to dissolve the test substance (Section 5.5.1), the volume used should not exceed the minimum necessary to dissolve and/or create a homogeneous suspension of the test substance. If the test substance is a mixture, formulation or a commercial product, none of the ingredients is considered a carrier unless an extra amount is used to prepare the stock solution.

(1b) The concentration of carrier used in the formulation of the test substance stock solution must be tested for potential biological effects (Section 5.6.1 and 5.6.3 (2c)).

(1c) Acetone at a final concentration of less than 10 mg/l is the preferred carrier.

5.6.3(2) Addition to Test Container

(2a) The test substance and carrier, if necessary as per Section 5.5.1, should be added to the microcosm in such a manner as to insure that the initial substance distribution is homogenous in the water column.

(2b) The test substance must be added to the microcosm tanks in the same manner as the initial add whenever there is a water exchange (Section 5.6.2(10)) but only in quantities sufficient to achieve the desired test concentrations in the replacement water.

(2c) The addition of a carrier to the selected carrier control tanks (Section 5.6.1(1)) must be performed in the same manner and at the same volume and frequency as is done in the exposure tanks.

5.6.4 Chemical Fate Sampling

(1) Water samples, to determine test substance levels in the water column (particulate and dissolved), should be taken immediately and at 1,3,6,12 and 24 hours after the initial addition. Thereafter, these samples should be taken prior to each water exchange.

(2) Water samples for chemical analysis must be collected: (a) While the turbulence (paddle) system is operating. (b) At a point at least three centimeters from the side of the tank and 10 centimeters below the air-water interface.

(3) Representative samples of the various zooplankton species should be collected once a week (see Section 5.6.5(4)) to analyze for uptake and accumulation of the test substance and, if possible, transformation products.

(4) A surface film, if formed by the test substance, should be

sampled prior to each water exchange and concentration determined by counting, with a liquid scintillation counter (LSC), the amount of radioactivity absorbed onto a filter pad.

(5) Potential losses of the test substance and transformation products to the atmosphere, either by volatilization or gaseous exchange, should be sampled weekly. Estimates of gas exchange rates with the atmosphere can be made by placing an inverted modified Pyrex crystallization dish (125 mm(diam) x 65 mm(depth)) 1-2 cm into the water (Figure 5) and drawing air from outside the test compartment through the dish at a designated rate and period of time. The air flow rate through the modified crystallization dish in each microcosm should be based upon the total lower surface area of the specific test compartment (Section 4.1.1(6),; Figure 1) and the total air flow rate through it. The air flow rate through the dish should be adjusted so that the rate per unit area equals that realized in the specific test compartment. The time interval used to measure gas transport should be of relatively short duration in order not to deplete the influence of the surface film trapped under the crystallization dish. Consecutive measures of gas transport should be made without repositioning the dish. If the rates of transport are the same, then the sampling time period is sufficient. If, however, the rates of transport decrease from the initial measure, then the time period is too long to obtain an accurate measurement.

(6) If the test substance is radiolabeled, estimates of the amount of test substance adsorbed onto the microcosm glass surfaces is accomplished by periodically removing some of the glass rods

through the slick protector (Section 4.2.1(7)), placing them in liquid scintillation vials, and counting them in a liquid scintillation counter.

If the test substance is not radiolabeled, direct measurements of the amount of test substance adsorbed onto the microcosm glass surfaces after 30 days will be determined by extracting all glass surfaces with a suitable solvent following removal of the seawater and sediment and then, measuring the quantities contained therein.

(7) All samples for chemical analysis should be processed immediately or be handled and stored appropriately to prevent losses by microbiological degradation, chemical reaction, volatilization, or adsorption.

(8) Standardized analytical methods should be used in performing the the analysis to develop chemical and physical data. Appropriate sources for such methodology include, but are not limited to AOAC, 1975; APHA, AWWA, and WPCF 1975; ASTM, 1979; USDI, 1977; USEPA 1974 and 1979. In general, atomic absorption analysis for metals and gas chromotography for analysis of organic compounds are preferable to colorimetric methods. However, in keeping with the recommendation that radiolabeled compounds be utilized for tracing the distribution of the test substances in the experimental microcosms, it is also recommended that liquid scintillation counting (LSC) be utilized for quantitation and that high pressure liquid chromotography (HPLC) be used for the concentration, separation and, if possible, characterization of the test substance and transformation products.

5.6.5 Ecological Effects Sampling

(1) During the operation of the definitive test, the plexiglass covers of the test facility (Figure 1) are installed and all biological and chemical samples collected through the removable sampling ports.

(2) All samples should be taken from approximately 10 cm below the water surface and away from the side of the tank. Sampling must be performed with the paddle system in operation.

(3) Biological sampling should always precede the water exchange on days when the exchange is scheduled.

(4) The following ecological information is considered to be essential:

(a) Phytoplankton abundance in numbers/ml per species.

(b) Zooplankton abundance in numbers/liter per life stage per species.

(c) Identification of all benthic organisms reported in numbers/m per species.

(d) Ammonia levels in µg at N/liter.

(e) Ammonia flux rates from the sediment in µg at $N/m^2/hr$.

Other or additional analyses such as bacteria counts or growth measures of benthic organism may be performed depending on conditions in the natural system being simulated and the analytical resources available to the investigators.

(5) The type of biological sample, volume of sample, collection frequency, method of collection and analysis are listed below.

(a) Phytoplankton samples are collected with an Eppendorf

pipette. Two milliliter samples are collected daily, counted and identified with methods described by Utermohl (1931).

(b) Zooplankton samples are collected twice weekly. A vacuum pump connected to a graduated separatory funnel which, in turn, is connected to the microcosm tank by a glass tube, allows a rapid non-destructive collection of two liters of water (Figure 6). The speed of sample collection must be great enough to overcome the avoidance reactions of the zooplankters. The two liter sample is passed through, and the organisms collected on, a 20 um mesh nylon screen. The organisms are rinsed into a Petri dish and preserved. Numbers and species composition are determined by examination under a dissection microscope. Wastewater from the two liter sample is pumped into a holding tank for subsequent removal of the test substance.

(c) Ammonia level in the water column is determined once a week. Twenty milliliter samples, collected with a syringe, are analyzed colorimetrically (Slawyk and MacIsaac 1972).

(d) Ammonia flux rates from the benthic box cores are measured weekly. Water flow to the benthic cylinders is halted for one to three hours (length of the incubation period is inversely related to temperature). Water samples of 20 milliliters are collected at the start and conclusion of the incubation period. Analysis for ammonia is identical to (c) above.

5.6.6 Procedure for Final Microcosm Sampling

(1) Those microcosm tanks that have been randomly selected for sacrifice and sampling at days 10 and 20 (Section 5.6.1(3)) and all remaining tanks following the exposure period must have the

tank and benthic box surface film (if present) removed and the water drained before taking the following samples.

(a) Triplicate sediment sub-cores (2.5 cm diam. x 7 cm deep) to determine the vertical distribution of the test substance in the benthic substrate. These cores include the sediment and associated biota.

(b) Identification and enumeration of all benthic organisms retained on a 0.5 mm screen.

(c) A representative sample of organisms from the benthic biota for analysis of the test substance and transformation products.

(d) Analysis of a number of the glass rods to obtain an estimate of the amount of test substance adsorbed to the microcosm glass surfaces. Test substance on the walls of two tanks and all associated glassware from each exposure treatment should be extracted, if possible, for confirmation of the uptake estimates obtained from the glass rods. If isotopes are not used, then all tanks receiving the test substance will be extracted for subsequent quantification.

5.7 Waste Disposal

5.7.1 Liquid wastes generated through sampling and water turnover must be collected and held until cleanup treatments have sufficiently reduced the chemicals and radioactivity to acceptable levels for disposal as per EPA and NRC (if using radio-labeled compounds) regulations.

5.7.2 Suggested techniques for waste water cleanup are:

(a) Filtration

(b) Activated charcoal

(c) Ion exchange

5.7.3 Contaminated solid wastes such as sediment, filters, charcoal, ion exchange resin, glassware, gloves, etc, must be packaged and disposed of in accordance with existing EPA and NRC regulations.

6. DATA ANALYSIS

6.1 Ecological Effects Analysis

Analysis of variance procedures (Steel and Torrie 1960) and regression techniques (Snedecor and Cochran 1980) should be used to analyze all data. The level of significance is set at the 5% level ($\alpha = 0.05$) unless otherwise defined. Documentation of all analysis used in the study must be provided.

6.1.1 Carrier Effects

Statistical comparisons between the carrier and sea water controls are necessary if a carrier, other than sea water, is used to add the test material to the experimental microcosms. The level of significance for this test is set at the 25% level ($\alpha = 0.25$).

(1) The method of repeated measures (analysis of variance) should be used to establish whether the carrier has an effect on all of the ecological variables measured with time. Depending upon the repeated measures analysis, means and variance for the entire 30-day experiment should be determined and examined for differences using a pairwise comparison procedure such as the Studentized Range Test (Snedecor and Cochran p. 234, 1980). For those variables which are observed at one time interval (e.g., numerical abundance of benthic species) a pairwise comparison

test should also be performed to detect differences between the carrier and sea water controls.

6.1.2 Microcosm Effects: Unperturbed Case

The type of comparisons for establishing the relationship of the control microcosms to the natural system should be based on the differences between the carrier and sea water (carrier-free) controls.

(1) If the responses from the carrier controls are statistically the same as that expressed in the sea water controls, then both control sets can be combined for purposes of quantifying the differences between laboratory controls and the natural system.

(2) If the responses from the carrier controls differs statistically from that expressed in the sea water controls, then only the sea water controls should be used to quantify the differences between laboratory controls and the natural system.

6.1.3 Test Material Effects: Perturbed Case

The model of repeated measures analysis of variance or a completely random design will be used to detect differences in the 30 day responses due to the test substance, relative to the laboratory control set. The specific replicates for the laboratory control set will depend on the results of 6.1.2.

6.2 Chemical Fate Analysis

The analysis should consist of two phases. First, performance of a budget of all radioactivity for each concentration of the test substance with statistical analyses to determine differences between the various compartments. Secondly, the radioactivity

measures are converted to weight of the test substance and breakdown products in the various compartments (Section 6.3) of the laboratory microcosms. The investigators should establish whether the means and associated variances of each variable are independent of one another. If dependence is found, then appropriate transformations (e.g., Log 10) should be applied so that the independence assumption can be satisfied. Statistical analyses are performed on the transformed data to test for differences in exposure concentrations.

6.2.1 Radioactivity Budgets

The computation of a complete budget of all radioactivity is required. The budget must include (a): the radioactivity added and/or removed either actively by investigator manipulation or gas transport, and (b) the distribution of radioactivity within the microcosms.

(1) Total Radioactivity Added

These additions consist of the amount of radioactivity added initially and all subsequent water turnover additions.

(2) Total Radioactivity Removed

Removals of radioactivity consist of water turnover losses (water and surface film) and losses to the gas phase.

(3) Radioactive Budget in Microcosm at Time t.

The following relationship will be used to budget the radioactivity in a microcosm at time, t:

Total activity in tank at time, t = (total activity added at time, t)- (total activity lost from tank at time, t)

Equation 1

and total activity added at time, t = (initial spike + $\sum_{o}^{\omega_t}$ (water turnover adds))

and

total activity lost from tank at time, t = ($\sum_{o}^{\omega_t}$ (water turnover aqueous losses) + $\sum_{o}^{\omega_t}$ (water turnover surface film losses) + $\sum_{o}^{t}$ (surface volatilization losses) + $\sum_{o}^{t}$ (gas transport losses))

where:

ω_t = discreet number of water turnover simulations at time, t

At the end of 30 day:

Total activity in tank = total glass surface activity + total dissolved water column activity + total particulate water column activity + total benthic biota activity (organisms not included in analysis of benthic sediment, Section 5.6.6).

Equation 2

(4) The sum of the measured activities from all compartments (equation 2) should be statistically compared to the calculated value derived from equation 1.

6.3 Fate of Test Substance and Transformation Products

For each concentration, determine the steady state total weight of the test substance and transformation products in the following microcosm compartments:

(a) Water column (particulate and dissolved fractions)

(b) Surface film

(c) Sediment core

(d) Glass surfaces

If the time to reach steady state is greater than 30 days for any of the compartments, use regression techniques to approximate the steady state concentration or weight per microcosm (e.g., asymptotic regression model, p. 395, Snedecor and Cochran 1980).

6.3.1 Fate Analysis

Test for concentration differences in the mean steady state weight of the test material and transformation products for each of the compartments listed in 6.3. In addition, test for compartmental differences in the mean steady state weights within each concentration.

6.4 Transport of Test Material and Transformation Products Determine the steady state weight of the test material and transformation products exported from the gas, liquid and surface film compartments. In addition, compute the total weight of the test material and transformation products exported during the experiment. If the time to reach steady state is greater than 30 days for any of the compartments, use regression techniques to approximate the steady state levels for each microcosm.

6.4.1 Transport Analysis

Test for concentration differences in the mean steady state weight or concentration of the test substance and transformation products for each of the compartments listed in 6.4. In addition

test for compartment differences in the mean steady state weight or concentration within each treatment condition. Perform the same analysis as above for the integrated weight of the test substance and transformation products transported during the 30-day experiment.

6.5 Bioconcentration

Compute both (a): the concentration of the test substance and transformation products, in either the organisms and/or their specific tissues, in units of weight/kg wet weight and (b): the ratio of animal or tissue concentration to medium concentration (Bioconcentration Factor). Choice of species should be based upon known or suspected linkages to man-based food chains. At least three replicates, (each from separate microcosm systems) of a given species/concentration combination should be measured. Indicate feeding type in the case of benthic species.

6.5.1 The choice of medium, for bioconcentration calculations, will be dependent upon the species feeding type. If the medium is sediment, then the mean vertical distribution of the test substance and transformation products in the sediment core should be used to define the exposure level.

6.5.2 Bioconcentration Analysis

Test for concentration differences within species.

6.6 Environmental Assessment of Test Material

Provide an environmental assessment of the potential fate and

effects of the test substance based on the analysis performed above and literature findings.

7. Test Protocol Bibliography

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List of Figures

a) Figure 1 Experimental Microcosm Facility

b) Figure 2 Paddle Drive and Speed Control System

c) Figure 3 Experimental Microcosm (not drawn to scale)

d) Figure 4 Air Control and Supply System for Benthic Pumps

e) Figure 5 Sampling Device for Gas Phase

f) Figure 6 Zooplankton Sampler

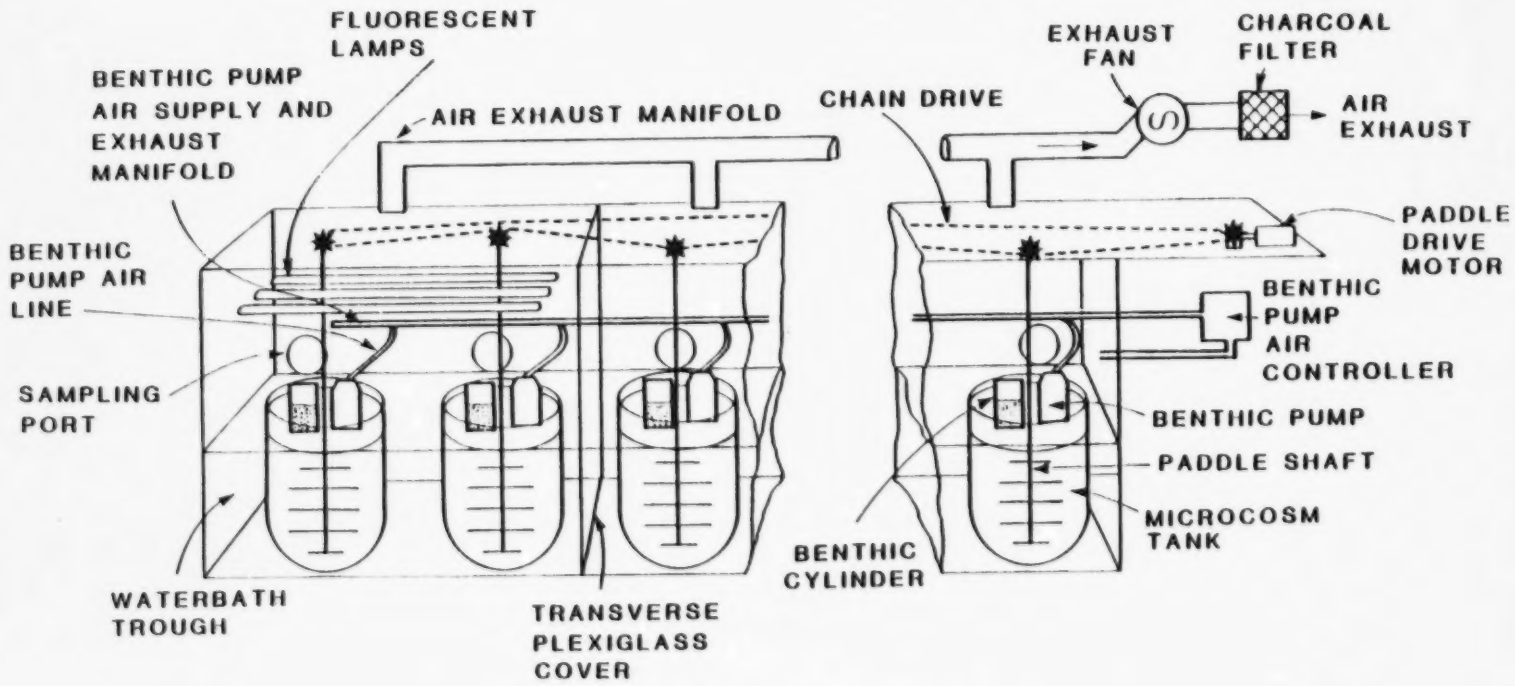

FIGURE 1. EXPERIMENTAL MICROCOSM FACILITY

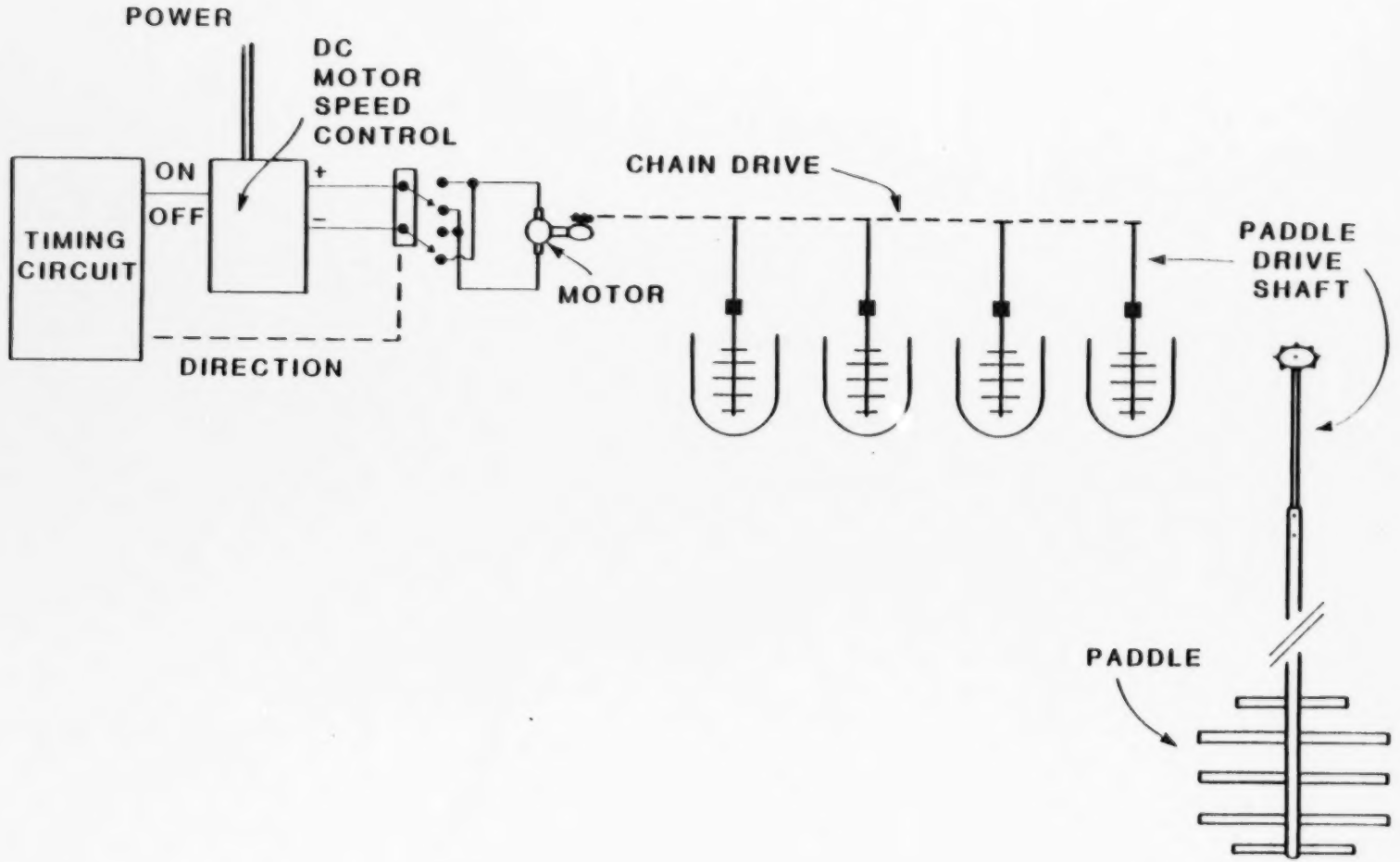

FIGURE 2.PADDLE DRIVE AND SPEED CONTROL SYSTEM

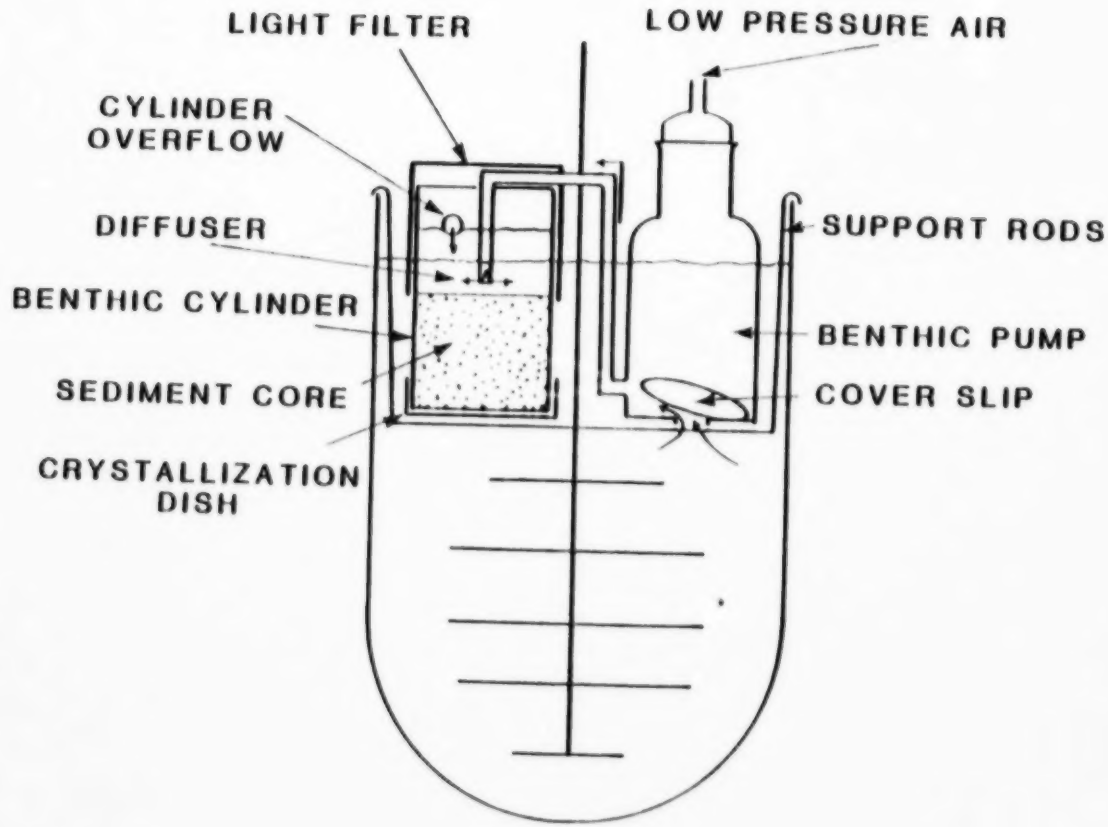

FIGURE 3. EXPERIMENTAL MICROCOSM (not drawn to scale)

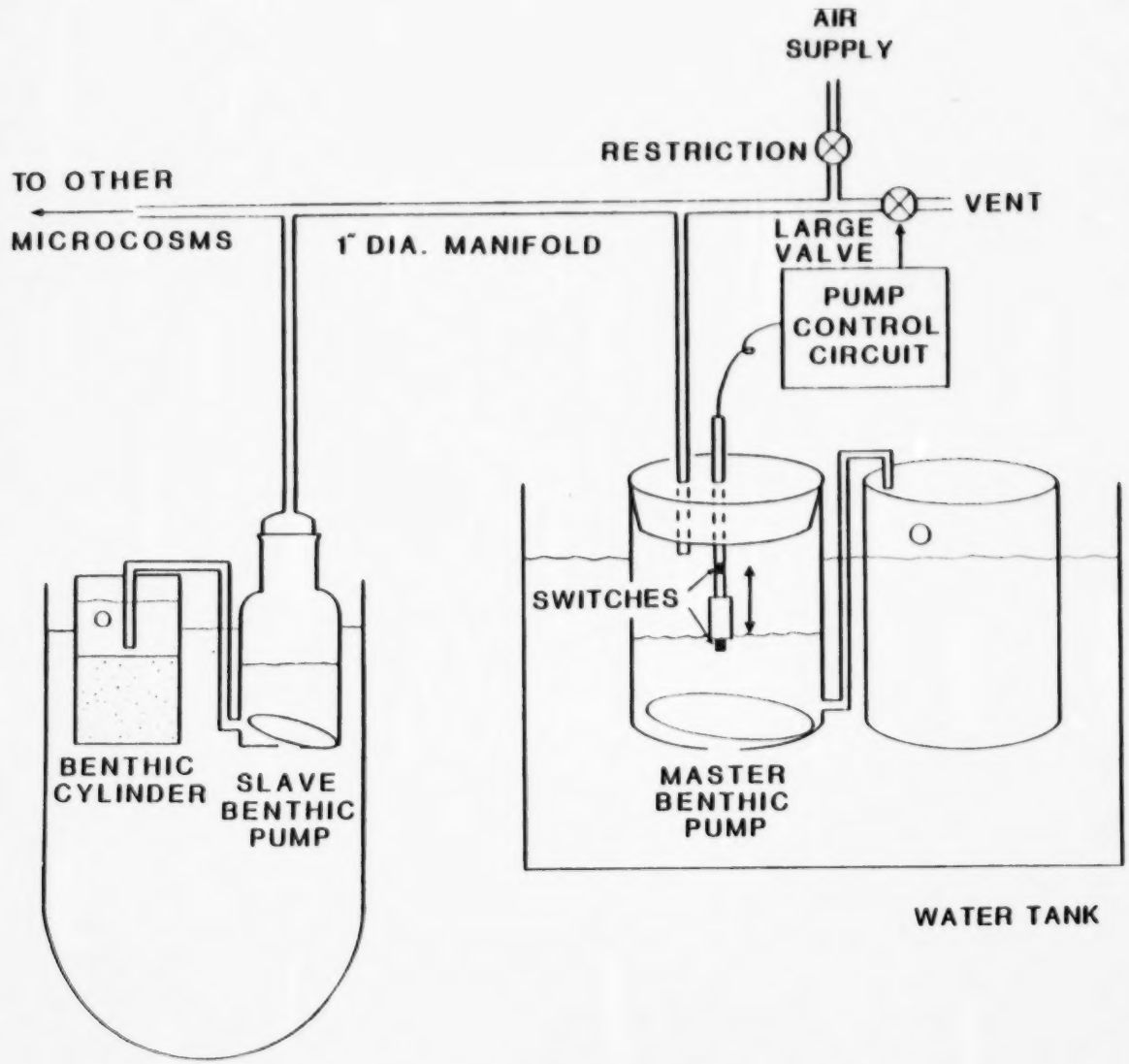

FIGURE 4. AIR CONTROL AND SUPPLY SYSTEM FOR BENTHIC PUMPS

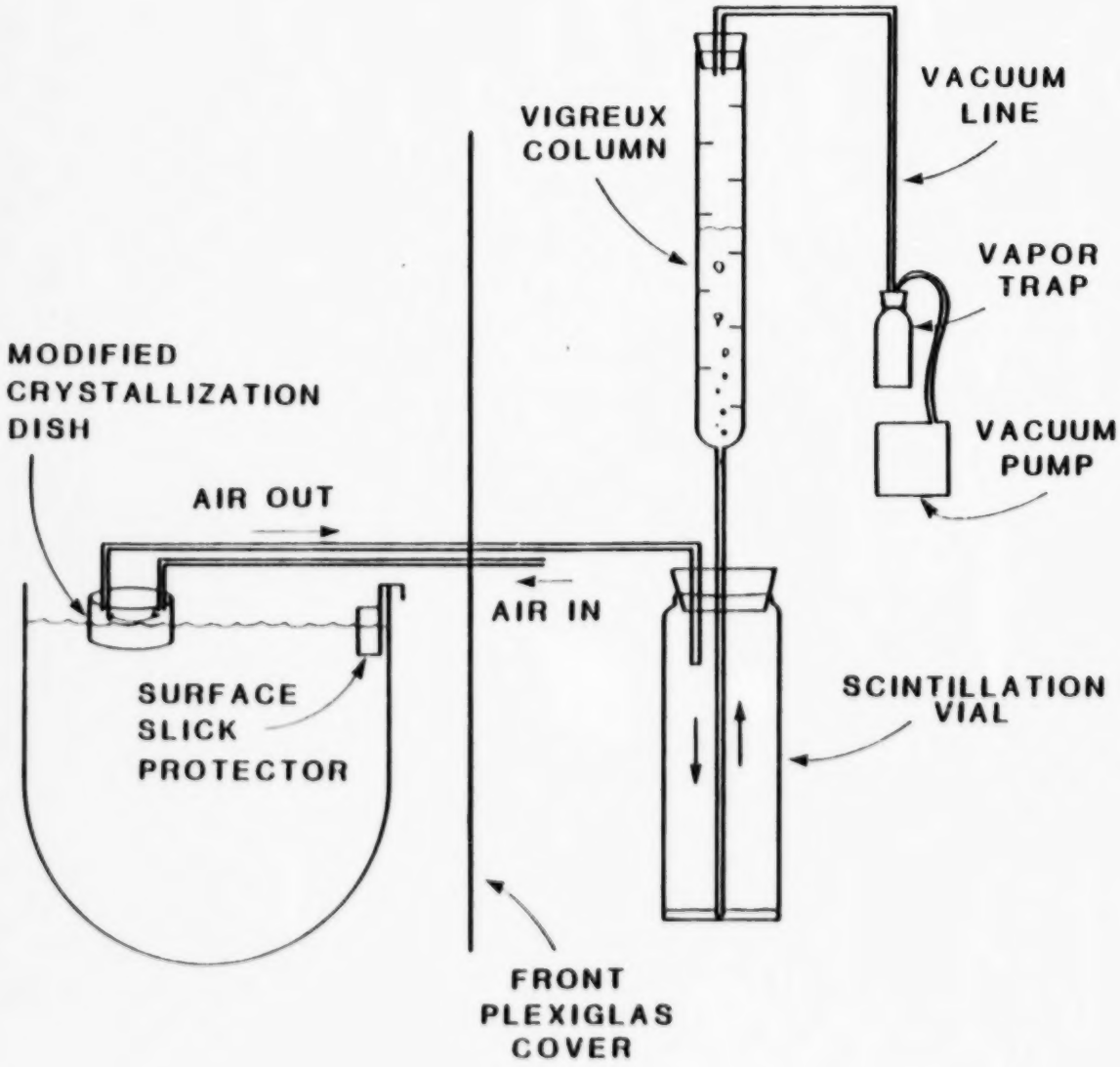

FIGURE 5. SAMPLING DEVICE FOR GAS PHASE

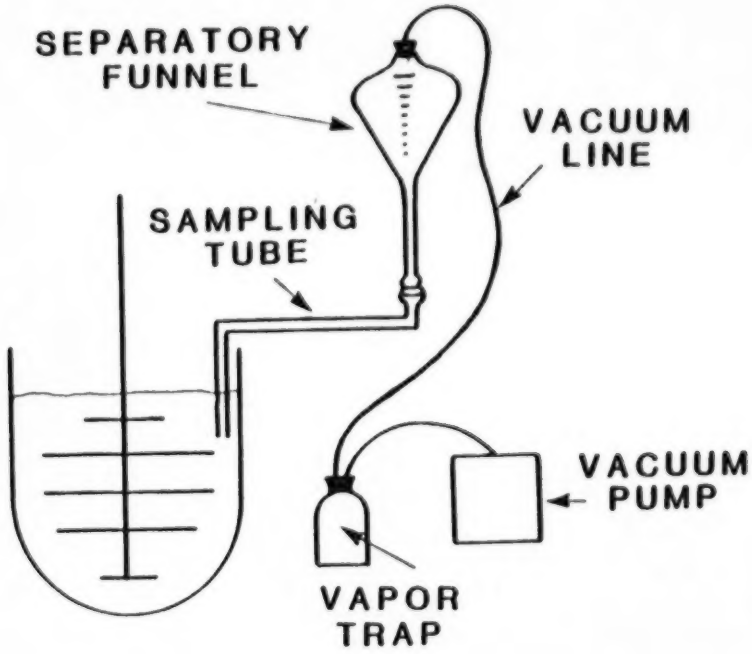

FIGURE 6. ZOOPLANKTON SAMPLER

SUPPORT DOCUMENT

(C)

Congressional Mandate

The U.S. Environmental Protection Agency is required by the Toxic Substance Control Act of 1976 (TSCA) to develop test standards which will provide information on a chemical's potential to affect human health and the environment.

Purpose of the Support Document

The purpose of the support document is to provide a general rationale for the specifications and use of the experimental microcosm system to determine the fate, ecological effects, and transport of selected test substances in coastal marine environments.

Rationale for the Use of Microcosms

The fate and ecological effects of a chemical introduced into a given ecosystem are necessarily dependent on both (a) the characteristics of the chemical and (b) the physical, chemical, and biological properties and interrelationships specific to that ecosystem. Any evaluation of potential environmental impact, therefore, must be based on data derived with these complexities in mind. Ideally the microcosm of a specific marine ecosystem should be as realistic as possible and allow for the (1) prediction of ecological effects and chemical fate at the system level of organization, (2) production of verifiable data, (3) standardization and replication among different laboratories, and (4) application of standard statistical techniques for data analysis.

The microcosm methodology described in the Test Protocol is used as an experimental, physical model of a specific portion of a natural ecosystem.

Much of the rationale for the design, conditions, and operation of the microcosm system is derived from attempts to duplicate the biotic and abiotic conditions of a natural system (Perez et.al., 1977). This experimental laboratory system meets the criteria listed above and can provide a predictive capability for assessing the potential fate and effects associated with the release of a specific chemical substance into coastal marine environments.

Rationale for Microcosm Design

Much of the rationale for the microcosm's materials and methods are stated in the test protocol or are intuitively obvious. These areas will not be covered in the support document.

Since the microcosm test protocol is intended to be a site specific test simulating a marine system, a number of system components and experimental conditions will be dependent on environmental conditions at the site (natural system) being simulated. The physical variables are light (intensity and photoperiod)(Section 5.6.2 (6)), temperature (Section 5.2.6 (7)), water turbulence (Section 5.6.2 (5)), water turnover rate (Section 5.6.2 (10)), and ratio of benthic surface area to water volume (Section 2.1.1 (6)). The biotic components are the endemic water column and benthic communities (Section 5.6.2 (1) and (3)).

In the microcosm, environmental conditions of photoperiod, temperature, turbulence, water exchange rate, benthic surface area, and benthic pumping rate are all established at levels that best duplicate conditions in the natural system. Previous experiments (Perez et al., 1977) have show that the field derived conditions are essential in order for the biotic responses of the microcosms to be equivalent to that in the natural system. Appropriate

light intensity, however, must be empirically determined.

The selection of tank volume of 140 liters (Section 4.2.1) is somewhat arbitrary. This size does, however, allow a representative benthic component when simulating most coastal and/or estuarine ecosystems. The primary consideration should be that the tanks be as large as possible while allowing for sufficient numbers of replicates within personnel, space, and/or monetary constraints.

Rationale for Microcosm Materials

"Hard glass" (semi-solid @ 600°) was chosen for all test system materials coming into contact with the exposure water (Section 4.2) because it was found to be the least adsorbtive material with regard to selected organic compounds (Davey, personal communication). This minimizes loss of the test substance to the tank surfaces and, therefore, provides more realistic exposure conditions. The amount of test material that does become adsorbed, however, can be quantified and easily removed following an experiment by (a) solvent extraction, for purposes of chemical fate quantification and (b) aci dstripping and/or ashing (450°C), for cleaning purposes.

Rationale for Test Procedures

Test water collection and distribution

The protocol requires the use of a water collection method which is non-destructive to planktonic organisms (Section 5.6.2) The application of such a method insures that any divergent behavior of the water column communities between the experimental microcosm and the natural system is due to microcosm effects and not the artifacts of the water collection and distribution methods (e.g., pumping).

The simultaneous filling of all microcosm tanks at the start of an experiment (Section 5.6.2.(2)) satisfies a major assumption of the recommended analysis of variance procedure (Section 6.1), specifically, that the experimental units (microcosms) are as identical as possible prior to the application of the treatment conditions (concentrations of the test substance).

Compound addition

The manner of preparation and addition of the test substance in a stock solution is dependent upon the nature of both the test substance and receiving water. In general, the methods prescribed in Standard Methods (1975) should be followed. If a carrier (Section 5.5.1) is used, the experimental design must include both carrier and carrier-free controls (Section 5.6.1 (1)). Acetone is suggested as the prefered carrier (Section 5.6.3 (1)) because (a) it is readily soluble in sea water, (b) it can dissolve a wide range of organic compounds of differing polarity, and (c) no ecological effects were observed in microcosm experiments using an acetone carrier at a level ten times greater than the recomended concentration (Perez et.al., In Press).

As per Section 5.5.2, it is recommended that test substance concentrations of 1, 10, 100 ug/l be used for the water column exposure concentrations and for subsequent water exchange additions. Although the choice of these concentrations is somewhat arbitrary, these levels often approximate or span the environmental concentrations of many anthropogenic substances in the field. The concentrations derived from existing range finding tests, utilizing one or more species (usually water column organisms), may not approximate the biotic responses expected from microcosm studies. For

example, Perez et.al. (In Press) showed that a water column exposure of 15.5 ug DEHP/l resulted in reduction of NH3 flux from the benthos. However, no ecological effects were observed on any of the water column organisms. Wilson et.al. (1978) was unable to detect any differences in the growth rate of the dinoflagellate, Gymnodinium breve when exposed to 100,000 mg DEHP/liter. Since range finding tests often do not incorporate a benthic community, the concentration at which an effect is observed may be grossly overestimated as was the case for DEHP.

Sampling

Simultaneous measurements of the test substance and ecological effects are prescribed for a thirty day period (Section 5.6.4 and 5.6.5). This sampling period enables the investigator to establish either the time for the test substance to reach quasi-equilibrium or the degree to which equilibrium is reached within the various physical and biotic compartments of the experimental microcosm. Furthermore, it is also possible to define the specific exposure concentration and body burden of the test substance at which ecological effects are expressed.

Ecological effects

Those measurements required to describe ecological effects (Section 5.6.5 (4)) are dependent on the characteristics of both the contained biotic communities and the test substance. Ideally, a structural and/or functional measure should be made from each trophic level. It is recommended that additional ecological measures be made in those compartments exhibiting significant accumulation of and/or exposure to the test substance.

Chemistry

Test substance compartments: The test substance is measured within the microcosms to establish (1) material budget (2) total transport and (3) bioaccumulation and potential trophic transfer (Section 5.6.4).

Bioaccumulation of the test substance can result in subsequent ecological effects and may also be a potential source for human exposure via direct consumption or upward trophic transfer. It is essential, from a management viewpoint, to be able to quantitatively define the relationship between the amount of test material added i.e., the input function, to subsequent bioaccumulation levels, trophic transfer, and ecological effects.

Analysis of sediment sub-cores is required to determine the vertical profile of the test substance and, thus, exposure concentrations for some of the benthic organisms.

It is necessary to develop a material budget of the test substance and resultant breakdown products throughout the course of the experiment (Section 6.2). This indicates the accuracy of measured concentrations and rates of removal within selected microcosm compartments. The compartments identified as essential to a material budget are the overlying gas phase, surface film (if produced), water column, sediment, glass surfaces and benthic macrofauna not included in the sediment sub-cores. Water analysis should include determination of test substance concentrations in terms of the total, dissolved, and particulate fractions.

The degree to which all the radioactivity, in the form of the test substance, added to the experimental microcosms can be accounted for is directly related to the validity of the amounts of the test substance reported in each of the

various microcosm compartments and biota. Therefore, it is essential to account for as much of the total radioactivity added as possible. This involves the measurement of the radiolabelled parent material as well as all breakdown products contained within and exported from the experimental microcosms via water turnover and gas phase (Section 6.2).

The amount of test substance found in the surface film and subsequently lost via gas transport or decomposition can, depending on the characteristics of the substance, form a significant fraction of the total budget. As a result, the design and performance of any chemical sampling program must take the contribution of the surface film into consideration (Section 6.2.1(3)).

The temporal dynamics and quantities of the test substance and breakdown products within selected microcosm compartments (Section 6.2.1(3)) enables the investigator to determine: (a) the exposure concentrations and the relationships to the body burdens of associated biota, (b) the concentration of the test substance at which ecological effects are observed, and (c) the total transport of the test substance and breakdown products. All of these measures and relationships can then be related to the three different quantities of the test substance added to the microcosms (season and the interaction of season and test substance, if performed).

Cleaning

The combination of benthic biological activity and water flow through the benthic chamber will result in significant but realistic quantities of suspended sediment in the microcosm tanks. Under natural conditions this

resuspended sediment settles back to the sediment surface. However, in the microcosms, the resuspended sediment settles to the bottom of the larger microcosm tanks. All such settled sediment is collected at the time of water turnover and returned to the benthic box (Section 5.6.2(9)) in order to compensate for and minimize the effects of this artifact.

Data Analysis

One of the major features of the recommended experimental design and statistical analysis (Section 6.1) is the abilitty to independently establish the quantitative effect of (1) the solvent carrier (if used) and, (2) the test substance for all the ecological variables measured. In Section 6.1.1, the error rate (α) was relaxed from 5 to 25% to compensate for the low replicate densities in carrier and carrier-free microcosms. By so doing, the ability to detect carrier effects is increased.

Furthermore, the degree of divergent biotic behavior between the control microcosms and the natural system provides a measure of the validity of the microcosm responses to the test substance.

Restrictions of Test Protocol

Scalar dimensions (Section 4.2.1) and simulation complexity (Section 4.1) limit the use and interpretation of results from experimental microcosms. Specifically, the size of the microcosm results in the exclusion of macrofauna such as fishes and large crustaceans. Since most, if not all, microcosms are so restricted, such a limitation is characteristic of this experimental approach.

In addition, the microcosm in its present design is restricted to "photo-stable" (Sections 1.1 and 5.1.1) test substances . Because the microcosm light intensity simulates sub-surface conditions of the natural system, the degree of breakdown of a "photo-unstable" test substance, due to

photolytic action, probably underestimates that which would occur in the natural system. If "photo-unstable" test substances are used, the interpretation and extrapolation of results to field conditions should be made with caution.

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